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THE NUTRITIONAL REQUIREMENTS OF ACETOBACTER. III. AMINO ACID REQUIREMENTS OF *ACETOBACTER XYLINUM*.*

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(Received for publication November 10, 1953)

The investigation of the amino acid requirements of *Acetobacter xylinum* is part of an over-all consideration of the factors that influence the efficiency of the conversion of ethyl alcohol to acetic acid in the production of vinegar. To that end, an understanding of the specific nutrient requirements of A. *xylinum*, and of other species of *Acetobacter* involved in the process, is important.

A. xylinum has not commonly been considered desirable for vinegar production because the zoogloeal formation by this organism clogs the acidifiers, thus impeding the vinegar process and facilitating the over-oxidation of the product (Shimwell, 1948). However, recent developments (Litsky and Goldman, 1953) have revealed that this organism may play an important part in synthesizing growth factors which can be utilized by the organisms active in vinegar production.

Comparatively little data have been reported on the nutritional requirements of the *Acetobacter* which are not involved in the production of vinegar. In an investigation of *A. suboxydans*, Underkofler, Bantz, and Peterson (1943) demonstrated that, beside a suitable source of carbon, organic nitrogen, and mineral salts, the medium must also contain pantothenic acid, *para*-aminobenzoic acid, and nicotinic acid. The amino acid requirements of *A. suboxydans* were determined by Stokes and Larsen (1945). Valine, isoleucine, alanine, and histidine were sufficient to support the growth of the organism; but growth was improved upon the addition of cystine or methionine. Ammonium sulfate alone did not support the growth of *A. suboxydans*, but showed a stimulatory effect when added to suboptimal concentrations of the required amino acids.

* Contribution No. 907 from the Massachusetts Agricultural Experiment Station.
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Foda and Vaughn (1953) reported that *A. melanogenum, A. rancens,* and *A. oxydans* required pantothenic acid, *para*-aminobenzoic acid, and nicotinic acid. Thiamin was required for *A. melanogenum* and *A. rancens,* but not for *A. oxydans.* Ammonium sulfate served as an adequate nitrogen source for *A. melanogenum,* but not for *A. rancens* and *A. oxydans.* Valine isoleucine, alanine, histidine, and cystine were necessary for the growth of *A. oxydans* and *A. rancens,* the latter also requiring glutamic or aspartic acid.

Very little information is available concerning the nutritional requirements of the *Acetobacter* involved in the commercial production of vinegar. It has been demonstrated by Hoyer (1898) and Beijerinck (1898) that *A. aceti* will grow in a medium containing mineral salts, ammonium nitrogen, and ethanol plus acetate as carbon sources (Hoyer's medium). Frateur (1950) has since shown that the acetate is unnecessary if sufficient phosphates are present in the medium. The growth of this organism in Hoyer's medium has been applied to the classification schemes of Visser't Hooft (1925) and Vaughn (1942) to separate *A. aceti* from the other species of *Acetobacter.*

Hoyer (1898), and Raghavendra Rao and Stokes (1953), have reported that *A. xylinum* will grow in a medium containing mineral salts, ammonium nitrogen, and glucose. The organism is not able to utilize the ammonium nitrogen when ethanol is the carbon source. Raghavendra Rao and Stokes (1953) have also shown that *A. xylinum* will develop in a glucose-salts-hydrolyzed casein medium. Litsky, Esselen, Tepper, and Miller (1953) have demonstrated that, for continued subculturing in a glucose-salts-hydrolyzed casein medium, *para*-aminobenzoic acid must be added to the medium.

Raghavendra Rao and Stokes (1953) have pointed out that, although Hoyer's medium and modifications of it are useful tools in taxonomic tests, the data obtained are of diagnostic value only and may not give a complete picture of the nutritional requirements of the strains of *Acetobacter.*

It has been observed by the authors that *A. xylinum* could not be sustained on continued transfers in a medium containing ammonium salts as a sole source of nitrogen. It is the purpose of this paper to determine the amino acids necessary for the maintenance of *A. xylinum* at a constant level of growth in a synthetic medium.

Materials and Methods

Organisms. The culture used in most of the experiments bears the identification *Acetobacter xylinum* Xl in the culture collection of the Bacteriology Department of the University of Massachusetts. A second culture was obtained from the National Institute of Health (*A. xylinum* NIH).

Media. Actively growing stock cultures were maintained on slants of yeast extract agar containing 0.5 per cent yeast extract (Difco), 0.3 per cent KH_2PO_4, 2.0 per cent glucose, and 2.0 per cent agar. The medium was adjusted to pH 6.0±0.2

The basal medium employed was a modification of the medium used by Litsky *et al.* (1953), composed of the substances listed in Table 1. The nitrogen source under investigation was added to this medium. The pH of all media was adjusted to be 6.0±0.2 after sterilization for 15 minutes at 15 pounds pressure.

TABLE 1

Basal Medium for the Nutritional Studies on *A. xylinum*

Components	Concentration per 100 ml medium
Glucose	3.0 g
p-Aminobenzoic acid	20.0 ug
Adenine sulfate	0.002 g
Guanine hydrochloride	0.002 g
Uracil	0.002 g
Mineral Salts A	
KH_2PO_4	0.05 g
K_2HPO_4	0.05 g
Mineral Salts B	
$MgSO_4.7H_2O$	0.02 g
NaCl	0.001 g
$FeSO_4.7H_2O$	0.001 g
$MnSO_4.3H_2O$	0.001 g

The liquid reference medium in all experiments contained Difco vitamin-free "casamino" acids (500 mg/100 ml medium) as a source of nitrogen.

The amino acids studied were of the highest purity obtainable commercially. They included alanine, beta alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lycine, methionine, phenylalanine, proline, serine, threonine,

tyrosine, tryptophane, and valine. These amino acids were made up in aqueous solutions, and when necessary, were acidified with HCl to effect solution. In the preparation of media, aliquots equal to 20 mg for L-amino acids and 40 mg for DL-amino acids were used per 100 ml of test medium.

Glassware. The 125-ml Erlenmeyer flasks in all experiments and all glassware in the preparation of media and inocula were acid-cleaned with chromic acid cleaning solution, rinsed six times in distilled water, followed by two rinses in double-distilled water.

Preparation of inocula. Inocula were obtained from actively growing stock cultures that had been transferred daily for at least three days. The cells were washed from the agar slant with sterile physiological saline (0.85 per cent) and were washed with saline three times. The washed cells were finally suspended in a 10-ml portion of saline in a test tube. The tube was shaken vigorously and then allowed to stand until the large clumps of cell settled out. One-tenth-milliliter portions of the supernatant were used to inoculate 10-ml quantities of the test medium in 125-ml Erlenmeyer flasks.

The inoculating procedure was necessitated because of the slimy growth of the *A. xylinum* cells brought about by the large amount of mucoid, cellulose, capsular material produced by the organism. Methods for the standardization of inocula could not be applied to this organism; therefore, consecutive experiments could not be compared on the basis of equivalent inocula.

Flasks were inoculated in duplicate and incubated at 30° C. for four days. The cultures in liquid media were shaken continuously in a shaking machine, during incubation, to provide proper aeration. The experiments were carried out for five successive transfers made at 24-hour intervals.

Determination of growth. Because of the slimy growth of the organism, the measurement of growth on the basis of turbidity was impossible. Growth was therefore estimated on the basis of total acidity. After incubation, the original 10 ml of medium, cells included, were titrated to a pH of 6.0 with N/100 NaOH. Because of the nature of the growth, no attempt was made to correlate the amount of acid produced with actual cell count.

The nutritional requirements of *A. xylinum* were determined by conventional methods.

Results and Discussion

The activities of the two strains of *A. xylinum* in response to synthetic media are summarized in Table 2. The data presented are representative of results obtained in repeated experiments.

TABLE 2
Activity of *A. xylinum* in Defined Media

Media	Ml N/100 NaOH to titrate to pH 6.0*	
	Strain XI	Strain NIH
Modified Hoyer's medium	**	**
Basal + $(NH_4)_2SO_4$ (0.1 per cent)	**	**
Basal + hydrolyzed casein (0.5 per cent)	52	37
Basal + 20 amino acids	44	32
Basal + isoleucine	20	**
Basal + isoleucine + valine or alanine	26	**
Basal + isoleucine + valine + alanine	30	26

* Average value for five serial subcultures.
** Could not be maintained for five serial subcultures.

In media containing ammonium salts as the sole source of nitrogen, it was observed that growth, as indicated by acid production, occurred for the first and, in some instances, the second transfer from non-synthetic media. However, in all cases where only ammonium nitrogen was supplied, it was found that the organism could not be maintained for five serial subcultures.

When hydrolyzed casein, or a mixture of 20 amino acids, was added to the basal medium, the activity of the organism remained relatively constant upon subculturing. The organism failed to grow when isoleucine was omitted from the amino acid mixture. It was observed that, although acids were produced by *A. xylinum* Xl in media containing (a) isoleucine and (b) isoleucine plus valine or alanine, the titration values varied considerably for the five transfers. When isoleucine, valine, and alanine were present in the medium, the titratable acidity was relatively constant for both strains of *A. xylinum* tested. Thus, it is believed that isoleucine, valine, and alanine constitute the smallest number of amino acids required for consistent results on continued subculturing.

Table 3 represents the results obtained in studies on the stimulatory

effect of amino acids in synthetic media. It is of interest to note that the activity of *A. xylinum* Xl in a medium containing the five amino acids, isoleucine, valine, alanine, glutamic acid, and methionine, increased the titratable acidity to the level obtainable with a mixture of 20 amino acids.

TABLE 3

ACTIVITY OF *A. xylinum* XL IN MEDIA CONTAINING VARIOUS COMBINATIONS OF AMINO ACIDS

Amino acids in basal medium	Ml N/100 NaOH to titrate to pH 6.0*
Isoleucine + valine + alanine + glutamic or aspartic acid (A)	36
(A) + histidine, tyrosine, phenylalanine or cysteine**	38
(A) + proline or arginine	41
(A) + glycine or serine	42
(A) + methionine	43
20 Amino acids	44
Hydrolyzed casein	52

* Average value for five serial subcultures.

** These amino acids were added to medium separately, but gave a common result.

No appreciable stimulation of acid production could be demonstrated upon the addition of $(NH_4)_2SO_4$ (0.1 per cent) to a medium containing isoleucine, valine, and alanine in the concentrations used in the determination of the amino acid requirements. When suboptimal concentrations of the amino acids were used, stimulation by $(NH_4)_2SO_4$ became apparent. This observation coincides with results observed with *A. suboxydans* (Stokes and Larsen, 1945) and with *A. rancens* and *A. oxydans* (Foda and Vaughn, 1953).

It has been the purpose of this study to determine the nutritional requirements of *A. xylinum*. Although it has been demonstrated that *A. xylinum* cannot survive repeated transfers in a glucose-salts-ammonium nitrogen medium, this medium, nevertheless, supports the growth of the organism for the initial transfer from nonsynthetic media. In the classification of *A. xylinum* in Bergey's Manual (6th ed.), and in the classifications of Visser't Hooft (1925) and Vaughn (1942), the development of the thick, zoogloeal, cellulose membrane on the surface of liquid media is one method of differentiating this organism from other members of the *Acetobacter*. Thus, a liquid

medium supporting growth for the development of the membrane is sufficient for the taxonomic test. It would seem that carry-over nutrients supplemented by the glucose-salts-ammonium nitrogen medium fulfill this requirement. This observation substantiates the view of Raghavendra Rao and Stokes (1953) that diagnostic media may not represent a complete picture of the nutritional requirements of the organism tested.

It has been demonstrated that *A. xylinum*, *A. suboxydans* (Stokes and Larsen, 1945), and *A. rancens* and *A. oxydans* (Foda and Vaughn, 1953) will not develop in the absence of organic nitrogen. When these organisms are grown in defined media, it can be observed that they share the common requirement for isoleucine, valine, and alanine. The differences observed are in the number of amino acids that must be added to maintain growth.

Summary

The amino acid requirements for two strains of *Acetobacter xylinum* were determined. The growth of the organism could be maintained for five serial subcultures in media containing glucose, mineral salts, essential growth factors, and a combination of isoleucine, valine, and alanine. The stimulatory effect of other amino acids was also determined.

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Growth, 1953, **17**, 201-214.

INHIBITION OF THE UTILIZATION OF GLUTAMINE BY LACTOBACILLUS ARABINOSUS[1]

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(Received for publication November 16, 1953)

INTRODUCTION

During the course of a study of the free amino acids of normal and cancerous tissues in mice, Roberts and Frankel (1949 and 1950) found the glutamine levels in tumors to be uniformly lower than in most of the normal tissues. It was considered that it might be feasible to attempt to disturb the protein metabolism of tumors by interference with glutamine metabolism. Most normal tissues, which contain relatively large quantities of free glutamine, would possibly be more resistant to such an interference. In order to test conveniently for possible glutamine inhibitors it was necessary to have reproducible systems in which small amounts of glutamine would have a marked effect on some growth processes. Ayengar, et al. (1951) subsequently described experiments in which microbiological systems employing *Lactobacillus arabinosus* were standardized for showing the enhancing effects of small amounts of glutamine on the utilization of D- and L-glutamic and α-ketoglutaric acids for growth. Ayengar and Roberts (1952a) found compounds which were competitive inhibitors of glutamic acid, but none of the substances tested inhibited the utilization of *isomolar* amounts of glutamine.

The present experiments describe the effects of a large number of substances structurally related to glutamic acid and glutamine on these microbiological systems when quantities of glutamine were employed which caused marked acceleration of growth, but which would support no growth in the absence of D- or L-glutamic acid or α-ketoglutarate.

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[2]Supported by a fellowship from the Charles F. Kettering Foundation. Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Materials and Methods

Compounds Used—The γ-L-glutamylmethylamide was furnished by Dr. F. C. Meyer of the Monsanto Chemical Co. 2-Acetylamino-1, 3,4-thiadiazole-5-sulfonamide was obtained from Dr. R. O. Roblin, Jr., of the American Cyanamid Co. All of the other analytically pure substances tested for glutamine inhibition were kindly given to us by Dr. Karl Pfister of Merck and Co., Inc. L-Glutamine and L-glutamic acid were purchased from General Biochemicals, Inc., and α-keto-glutaric acid from Nutritional Biochemicals Corporation. D-Glutamic acid was purchased from Amino Acid Manufactures of the University of California.

Assay Methods—The microbiological procedures using *L. arabinosus* 17-5 have all been described previously by Ayengar, et al. (1951) and references to the original literature given. Assays were performed at pH 7.0 in a high aspartic acid medium (Medium 1), under which circumstances there is a sharp differentiation in response to glutamic acid and glutamine presumably because of the limitation of the ability of the organism to synthesize glutamine, and at pH 6.0 in a low aspartic acid medium, (Medium 2), in which growth occurs equally well with glutamic acid or glutamine. Solutions of all of the substances tested and glutamine were sterilized by Seitz filtration. In all instances readings of turbidity were made at several time-intervals.

Experimental

Influence of Glutamine on Response to Glutamic Acid on Medium 1—The system in which glutamine enhances the rate of utilization of L-glutamic acid for growth on a high aspartic acid medium at pH 7.0 was chosen for initial screening purposes. Figure 1 shows a 3-dimensional plot of the time-course of growth with 75 γ of glutamic acid alone and in the presence of varying amounts of glutamine. The final growth attained in all tubes was the same regardless of the amount of glutamine, when the latter substance was present in quantities up to 6 γ per tube. The time-lag in growth observed in the absence of glutamine was largely eliminated in the presence of 1 γ of glutamine and a maximal response was shown with 2 γ of glutamine. In the experiments described in the following section all tubes contained 75 γ of glutamic acid and 1 γ of glutamine.

Screening of Compounds for the Inhibition of the L-Glutamic Acid-Glutamine System—Employing the above assay system all of the sub-

stances discussed below were tested in duplicate rows of tubes in concentrations of 2, 4, 6 and 8 μM per tube in a final volume of 1.5 ml. All of the compounds found to exert either an inhibitory or enhancing effect on the rate or extent of growth were retested and subjected to further experimentation. The compounds tested will be discussed by groups.

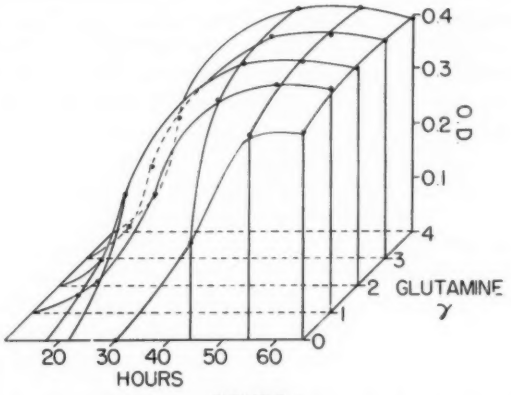

FIGURE 1

Time-course of growth with 75 γ of glutamic acid alone and in the presence of varying amounts of glutamine.

Group I—γ-Glutamylamides—Tests were performed on γ-L-glutamylmethylamide, γ-DL-glutamylethylamide, γ-DL-glutamylbutylamide, γ-DL-glutamylbenzylamide, and γ-DL-glutamylanilide. Only the γ-L-glutamylmethylamide was found to be markedly inhibitory. However, a pronounced inhibition at the earlier time-intervals was followed by a remarkable enhancement of growth at later times. A more detailed study of this phenomenon will be presented subsequently.

Group II—γ-Glutamyl Esters—All of the three esters tested enhanced the growth in this system. The following amounts of growth in percentages of the controls, were found in the presence of 8 μM of the compounds at 43 hours, a time at which maximal growth was obtained in all tubes: γ-methyl-L-glutamate, 232; γ-ethyl-L-glutamate, 158; γ-benzyl-DL-glutamate, 120. 4 μM of the methyl ester gave as great an effect as did 8 μM. A large series of supplementary experiments revealed that the γ-methyl-L-glutamate could not replace glutamic acid for growth in isomolar amounts and that equivalent

amounts of this compound could not replace glutamine in any of the glutamine-enhancing systems. The mechanism by which the γ-glutamyl esters exert their effect is under further investigation.

Group III—Hydrazides and Substituted Hydrazides—The most effective compounds in this group were γ-L-glutamylhydrazide and acetone-γ-L-glutamylhydrazone, compounds previously found to be effective antagonists of glutamine utilization for some strains of hemolytic streptococci by Roper and McIlwain (1948). β-DL-Aspartylhydrazide, a compound also developed by the latter workers, was somewhat less effective than the above 2 compounds, while no significant inhibition was found with the following substances: N-benzoyl, N′-γ-DL-glutamylhydrazine; N,N′-bis(γ-L-glutamyl) hydrazine; L-pyrrolidone carbohydrazide; α-(N-phenacetyl)-γ-L-glutamyl hydrazide. The above results show clearly that the presence of a hydrazide grouping is not sufficient for inhibition, the most active substances being those most closely related to glutamine.

Group IV—α-Methyl Compounds—α-DL-Methylglutamic acid was the most potent inhibitor of this group. Previous reports have been made of inhibition of bacterial growth (Ayengar and Roberts (1952a)) and glutamic decarboxylase (Roberts (1953)) by this compound. The inhibitory properties were destroyed by substitution on the α-amino group as evidenced by the lack of activity of carbamyl-α-DL-methylglutamic acid and acetyl-α-DL-methylglutamic acid. α-DL-Methylasparagine was at most only very weakly active, while α-DL-methylaspartic acid was ineffective as an inhibitor. α-DL-Methylmethionine sulfoximine had much weaker activity than methionine sulfoximine itself.

Group V—Miscellaneous Cyclic Compounds—None of the compounds in this group had any inhibitory properties. The compounds tested included the following: DL-pyrrolidone carboxylic acid; L-pyrrolidone carboxamide; ethyl-L-pyrrolidone carboxylate; 6-hydroxy dihydropyridazine-3-carboxylic acid; pyrrole-2-carboxylic acid; 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide; and 2-methyl-3,4-dihydro-Δ′-pyrrolonine-5-carboxylic acid hydrochloride.

Group VI—Miscellaneous Straight-chain Compounds—Only β-hydroxy-DL-glutamic acid and DL-methionine sulfoximine proved to be inhibitors in this group. The following substances gave negative results: Allo-β-hydroxy-DL-glutamic acid; α-γ-diaminoglutaric acid; α-ketoglutaric, O-methyloxime; α-DL-amino-ε-hydroxy caproic acid;

and methionine sulfoxide, a compound found to be an antimetabolite of glutamic acid for *L. arabinosus* by Borek and Waelsch (1949). The observed ineffectiveness of methionine sulfoxide is probably explained by the very high ratio of inhibitor to metabolite required for inhibition in the test system of Borek and Waelsch even in the absence of added glutamine. Since all the tubes contained one gamma of glutamine much larger amounts of methionine sulfoxide than the maximum of 10μM employed in the present studies would be required to produce inhibition. Some previous studies have been made of the two racemic diastereoisomeric modifications of β-hydroxyglutamic acid by Ayengar and Roberts (1952) and Umbreit and Heneage (1953), and of a mixture of the racemates by Borek and Waelsch (1949) and of methionine sulfoximine by Newell and Carman (1950).

Comparison of Inhibitory Potencies on the Glutamic Acid-Glutamine System in Medium 1—The numerical results for the experiments with the compounds which were found to be inhibitory are shown in Table 1. Six of the compounds listed in Table 1 and α-DL-methyl-

TABLE 1

INHIBITION OF THE L-GLUTAMIC ACID-GLUTAMINE SYSTEM IN MEDIUM 1

Results are expressed as percentages of control growth at the time when the control tubes first attained maximal turbidity (40-43 hours)

Compound tested	Percentage of normal growth μM of inhibitor per tube			
	2	4	6	8
γ-L-Glutamylhydrazide	3	3	3	0
Acetone-γ-L-glutamylhydrazone	18	3	3	0
β-Hydroxy-DL-glutamic acid	52	23	11	6
β-DL-Aspartylhydrazide	61	42	21	15
γ-L-Glutamylmethylamide	58	45	28	25
DL-Methionine sulfoximine	85	71	59	52
α-DL-Methylglutamic acid	100	61	64	33

asparagine were then tested for inhibition at 5 and 10 μM levels in experiments performed at the same time in order to minimize the experimental variations. The time-courses of growth in the tubes having 10 μM of inhibitor are shown in Fig. 2. As in the experiments in Table 1, the γ-L-glutamylhydrazide (GGH), acetone-γ-glutamyl hydrazone (AGH), and the β-hydroxy-DL-glutamic acid (BHG) were the most effective inhibitors. α-DL-Methylglutamic acid (AMG) and methionine sulfoximine (MS) were weaker inhibitors, while γ-L-glutamylmethylamide (GMA) showed its typical behavior, which was previously described. α-DL-Methylasparagine was relatively in-

effective in this system. In further experiments it was shown that the above inhibitors possessed the same relative potency on DL-glutamic acid as on L-glutamic acid, although the utilization of DL-glutamic acid was much less efficient under these conditions as evidenced by a longer lag period in the control tubes and a smaller extent of final growth.

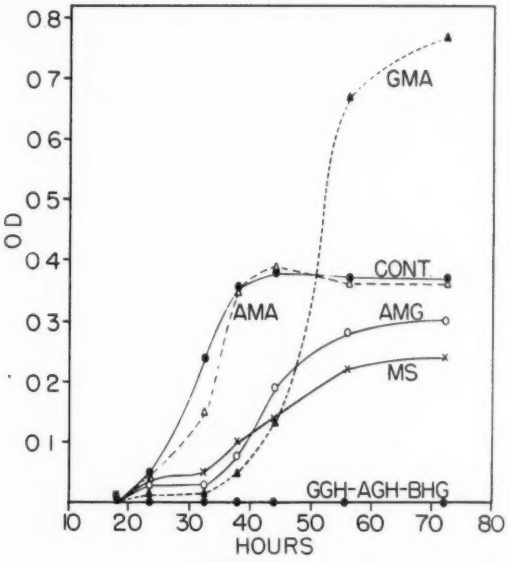

FIGURE 2

Inhibition of the L-glutamic acid (75 γ)—glutamine (1 γ) system in Medium 1 by 10 μM quantities of γ-L-glutamylhydrazide (GGH), acetone-γ-L-glutamylhydrazone (AGH), β-hydroxy-DL-glutamic acid (BHG), α-DL-methylglutamic acid (AMG), methionine sulfoximine (MS), γ-L-glutamylmethylamide (GMA), and α-DL-methylasparagine (AMA).

Reversal by glutamine of inhibition by GGH, AGH and BHG—The results in Table 2 show that small amounts of glutamine can reverse the inhibition produced by the above compounds when present to the extent of 10 μM per tube. Smaller amounts of glutamine are required to produce a reversal of the effects of BHG than of GGH and AGH, a result in keeping with the somewhat greater inhibitions produced by the latter two compounds in the original test system.

Further experiments on effect of γ-L-glutamylmethylamide on the glutamic acid-glutamine system—Since 2-dimensional paper chroma-

TABLE 2
REVERSAL WITH GLUTAMINE OF INHIBITION OF GROWTH BY GGH, AGH, AND BGH*

Glutamine γ/tube	Time hrs.	Growth (Optical density) Control	GGH	AGH	BHG
0	22	0.	0	0	0
	44	0.18	0	0	0
	55	0.38	0	0	0
	67	0.40	0	0	0.02
1	22	0.06	0	0	0.03
	44	0.40	0	0	0.05
	55	0.43	0	0	0.06
	67	0.42	0	0	0.07
2	22	0.18	0	0	0.06
	44	0.42	0.01	0	0.12
	55	0.42	0.05	0	0.26
	67	0.41	0.34	0	0.38
3	22	0.18	0	0	0.07
	44	0.42	0.06	0.03	0.24
	55	0.42	0.32	0.17	0.40
	67	0.40	0.37	0.35	0.46
4	22	0.20	0.02	0.01	0.12
	44	0.42	0.20	0.23	0.44
	55	0.43	0.37	0.35	0.45
	67	0.41	0.37	0.38	0.46

* 10 μM of inhibitors/tube employed. GGH, γ-L-glutamylhydrazide; AGH, acetone-γ-L-glutamylhydrazone; BHG, β-hydroxy-DL-glutamic acid. All tubes contained 75 γ of L-glutamic acid in Medium 1.

tography of large amounts of GMA failed to reveal even a trace of ninhydrin-reactive impurity, the accelerating effect on growth at later time-intervals could not be attributable to contamination with glutamine or glutamic acid. Further experiments with GMA showed that 5 μM of this compound in the presence of 1 γ of glutamine did not support any growth. This makes it seem unlikely that the GMA is split rapidly to yield glutamic acid and methylamine, since under these circumstances it would be expected that large amounts of GMA would be able to support growth in the absence of added L-glutamic acid. When experiments were performed with 75 γ of L-glutamic acid and 1 γ of glutamine per tube in the presence of 1 to 5 μM of GMA, the rate of growth was decreased progressively at earlier time intervals, a maximal effect being found at the 4 μM level (Fig. 3). However, at all concentrations of GMA the growth eventually exceeded that found in the controls, attaining approximately twice the control levels in the tubes containing 2-5 μM. In similar experiments with 2-10 μM of γ-DL-glutamylethylamide or γ-DL-glutamylbutylamide no effect

was observed on the growth pattern, curves identical with the controls being observed at all concentrations. The results in Fig. 4 show clearly that the ultimate enhancement of growth by GMA is not related to the inhibition found at earlier time intervals, since the inhibition can

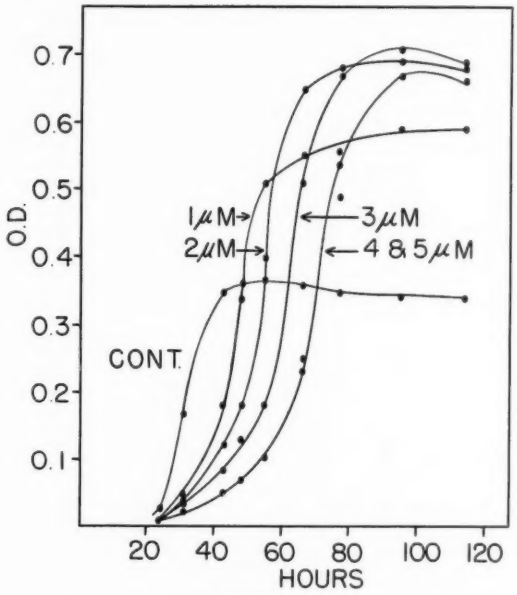

FIGURE 3

Influence of varying quantities of γ-L-glutamylmethylamide (GMA) on growth on L-glutamic acid (75 γ)—glutamine (1γ) in Medium 1.

be completely overcome by glutamine, while the enhancement persists. The above results suggest that the nitrogen of GMA can be used by the bacteria. It is interesting that a purified liver enzyme studied by Meister and Tice (1950) which can catalyze the transamination of a variety of keto-acids with glutamine was also found capable of transaminating GMA, but not γ-glutamylethylamide. Numerous experiments, however, performed under a large variety of conditions with intact cells, vacuum dried preparations, and acetone powders have so far failed to reveal a similar enzyme in *L. arabinosus*.

Inhibition of utilization of L-glutamic acid on Medium 2—Since only small amounts of glutamine are necessary to overcome the in-

hibition of AGH and GGH (Table 2) it would be expected that these substances would only be good inhibitors of glutamine utilization under conditions which restrict glutamine synthesis, such as exist in Medium 1, and poor inhibitors of growth when the ability

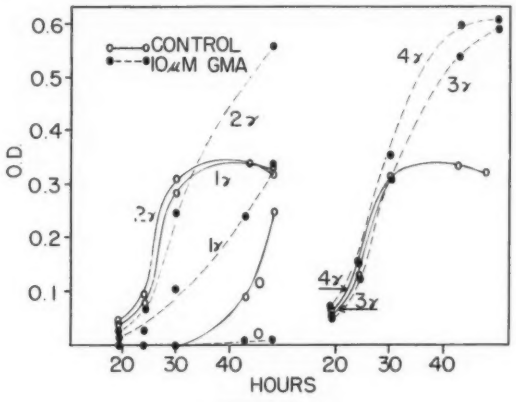

FIGURE 4

Influence of γ-L-glutamylmethylamide (GMA) on growth on L-glutamic acid (75 γ) in Medium 1 in the presence of varying amounts of glutamine (0-4 γ per tube).

of the bacteria to synthesize glutamine is unhampered, as when Medium 2 is employed. It was shown previously by Ayengar and Roberts (1952) that AMG and BHG were good inhibitors of the utilization of glutamic acid probably acting by inhibiting the synthesis of glutamine and that the inhibition was competitive with glutamic acid. An experiment was performed in which the inhibition by various concentrations of the latter two substances was compared with that shown by AGH and GGH on Medium 2 in the absence of added glutamine. Typical results are shown in the plots of the time-course of growth for the tubes containing 8 μM of compound (Fig. 5). BHG was the most potent inhibitor of the substances tested, only approximately 3% of the control growth being attained at 41 hours. Although the time-lag of first appearance of growth was increased by both AGH and GGH, the inhibition was eventually overcome and a normal amount of final growth found. However, the growth attained at the last period of measurement in the presence of AMG was considerably less than that in the control tubes or in those containing AGH or GGH.

It may, therefore, be concluded that in the absence of glutamine but under conditions of growth which allow rapid synthesis of glutamine from glutamic acid those substances that can inhibit the formation of glutamine from glutamic acid are more effective inhibitors than

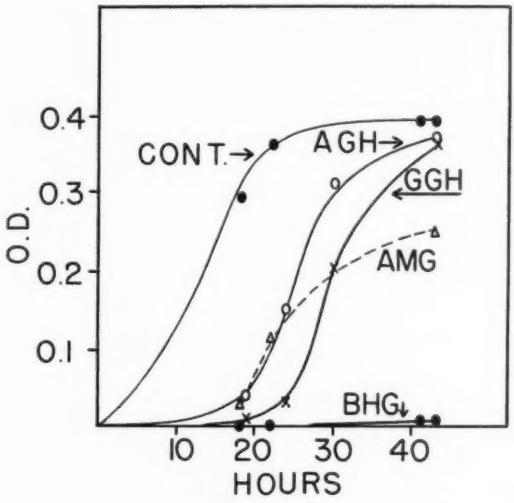

FIGURE 5
Inhibition of utilization of L-glutamic acid (75 γ) on Medium 2 by 8 μM quantities of inhibitors.

those which inhibit the subsequent utilization of glutamine for growth. Of course, AMG and BHG also may inhibit reactions of glutamic acid other than those involved in glutamine production.

Inhibition of growth on the D-glutamic acid and α-ketoglutarate systems in Medium 2—Since the previous experiments of Ayengar, et al. (1951, 1952b) have shown that small amounts of glutamine can enhance the growth of *L. arabinosus* on D-glutamic acid and on α-ketoglutarate in a medium from which L-glutamic acid was omitted, experiments were performed to determine whether the compounds shown to be inhibitory for growth on L-glutamic acid in the preceding sections would also inhibit the utilization of D-glutamic acid and α-ketoglutarate. Varying quantities (2-10 μM) of the inhibitors were added to tubes containing 150 γ of D-glutamic acid

and 2 γ of glutamine or 2.5 mg. of α-ketoglutarate and 2 γ of glutamine in Medium 2. The omission of glutamine always resulted in a pronounced lag in growth with D-glutamic acid and the failure of any growth to take place in the α-ketoglutarate system.

The results for the compounds tested for inhibition of utilization of D-glutamic acid are shown in Table 3. At later time-intervals

TABLE 3

INHIBITION OF THE D-GLUTAMIC ACID-GLUTAMINE SYSTEM IN MEDIUM 2

Results are expressed as percentages of control growth at the time when the control tube first attained maximal turbidity (44-50 hours)

Compound tested	Percentage of normal growth μM of inhibitor per tube				
	2	4	6	8	10
β-Hydroxy-DL-glutamic acid	2	2	2	2	0
DL-Methionine sulfoximine	10	8	6	4	2
α-DL-Methylglutamic acid	48	24	14	8	6
γ-L-Glutamylhydrazide	62	60	58	55	55
Acetone-γ-L-glutamyl hydrazone	65	59	59	58	56
γ-L-Glutamylmethylamide	82	80	78	74	76

there was an acceleration of growth above the controls in the presence of GMA. In other experiments it was shown that the inhibition by GGH and AGH eventually was completely overcome, while that by BHG, MS, and AMG persisted for as long as 85 hours.

Also with α-ketoglutarate BHG was found to be the best inhibitor, while MS and AMG were also found to be considerably more inhibitory than AGH and GGH (Fig. 6). Since BHG was a potent inhibitor of growth on α-ketoglutarate even in the presence of 30 γ of glutamine (Ayengar and Roberts 1952a) and since only small amounts of glutamine are necessary for complete reversal of the inhibition by large amounts of BHG on Medium 1 in the presence of 75 γ of L-glutamic acid (Table 2), it appears unlikely that BHG acts on the α-ketoglutarate system by inhibiting glutamine synthesis or the reaction (or reactions) by which glutamine sparks growth on L-glutamic acid. It appears likely, therefore, that BHG inhibits synthesis of glutamic acid from α-ketoglutarate, a process in which glutamine may participate catalytically (Ayengar, et al. 1951). GMA accelerated growth at all times (Fig. 6). Further studies of the latter finding have been reported elsewhere by Roberts, et al. (1954).

Discussion

Examination of the large literature dealing with the role of glutamine in growth in plants, animals, and microorganisms in reviews by Archibald (1945) and Waelsch (1952) reveals that none of the known enzymatic conversions of glutamine can be employed to explain the observed effects at the present time. The delineation of

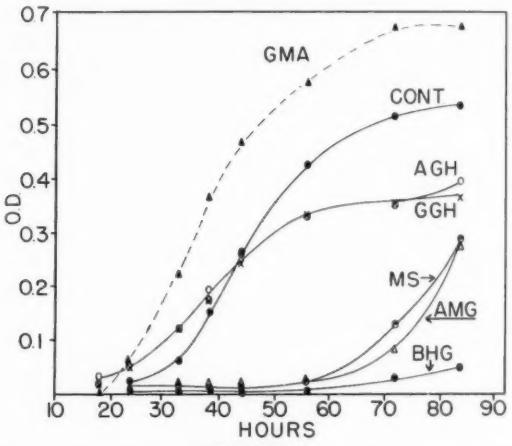

FIGURE 6

Inhibition by 10 μM of inhibitors of growth on the α-ketoglutarate (2.5 mg)—glutamine (2 γ) system in Medium 2.

growth systems in synthetic media in which small amounts of glutamine have a marked enhancing effect in the utilization of L-glutamic and D-glutamic acids and α-ketoglutarate for growth by *L. arabinosus* and the finding of a series of inhibitory metabolites for these systems will be of considerable value in studies of the mechanism of glutamine action. The experiments in the present communication have indicated the general areas in which the growth-inhibitory substances may act. The difficulties in determining the exact locale of the action of a metabolic inhibitor from experiments dealing with growth of bacteria have been summarized forcefully by Kihara and Snell (1952). Studies are now in progress in which a correlation is being made between growth inhibition and the influence on those enzyme systems of *L. arabinosus* which might play a role in the utilization of glutamic acid and related metabolites for growth.

Summary

A large series of compounds structurally related to glutamic acid and glutamine was tested for inhibition of growth of *Lactobacillus arabinosus* under conditions in which L-glutamic acid was the limiting metabolite and small amounts of glutamine produced a marked acceleration of growth without affecting the final amount of growth attained.

γ-L-Glutamylhydrazide (GGH), acetone-γ-L-glutamylhydrazone (AGH) and β-hydroxy-DL-glutamic acid (BHG) were the most effective inhibitors of the above system, while α-DL-methylglutamic acid (AMG) and methionine sulfoximine (MS) were somewhat less effective. It was shown that small amounts of glutamine could reverse inhibition by GGH, AGH, and BHG.

γ-L-Glutamylmethylamide (GMA) produced an inhibition at earlier time-intervals which was followed by marked enhancement at later time-intervals. Correlative experiments suggested that the inhibition by GMA might result from a competition with glutamine, while the enhancing effect might be related to utilization of the nitrogen of GMA.

γ-Methyl-L-glutamate, γ-ethyl-L-glutamate and γ-benzyl-DL-glutamate also stimulated growth, but could not support growth in the absence of L-glutamic acid.

AMG and MS were found to be more inhibitory than GGH and AGH in experiments with L-glutamic acid carried out in such a way that the synthesis of glutamine was not limited by conditions in the medium.

BHG, MS, and AMG proved to be better inhibitors than GGH and AGH in experiments in which growth was studied with D-glutamic or α-ketoglutaric acids in the presence of small amounts of glutamine at pH 6 in a low aspartic acid medium.

Of the substances studied, BHG was most consistently inhibitory under the various conditions employed for testing.

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MODIFICATIONS[1] IN A CALLUS CULTURE OF *SEQUOIA SEMPERVIRENS* AFTER GROWTH ON P^{32} AND S^{35}

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After the earlier finding (Ball, 1953b) that the radioisotopes P^{32} and S^{35} at the concentration of 1.5 μc/ml. of medium caused the death of the calluses, it was desirable to study their growth at the approximate maximum nonlethal dosage. It was important to determine whether the cells of this callus show differential susceptibility to the radioisotopes. Furthermore, such a study might yield some data on the process of differentiation. If it were possible to grow the callus in a concentration of radioisotope that would kill or prevent the growth of all but one type of cell, a culture might be obtained from the latter. It would be of great theoretical interest to determine whether such a culture would be of one cell type, or whether the single initial type of cell would, by processes of differentiation, produce other types of cells in subsequent growth.

In this callus that consists mostly of parenchyma, and where new cells are formed by distinctive meristems, it is important to know which cells are most susceptible to internal radiation, and which cells are sufficiently resistant to the radiation to be able to continue cell division.

This callus has the following types of cells (Ball, 1950): mature parenchymatous (some of which have tannins), meristematic cells at margins of the callus, and meristems forming cambia around nests of tracheids and phloem-like cells. The form of the callus is normally a thin crust of greenish-brown tissue that grows over the surface of the agar when the latter contains autoclaved sucrose. It was a question of some interest to determine whether those cells surviving internal radiation would carry on, by their cell divisions, the normal pattern of growth.

Materials and Methods.—Attempts to grow the callus on media

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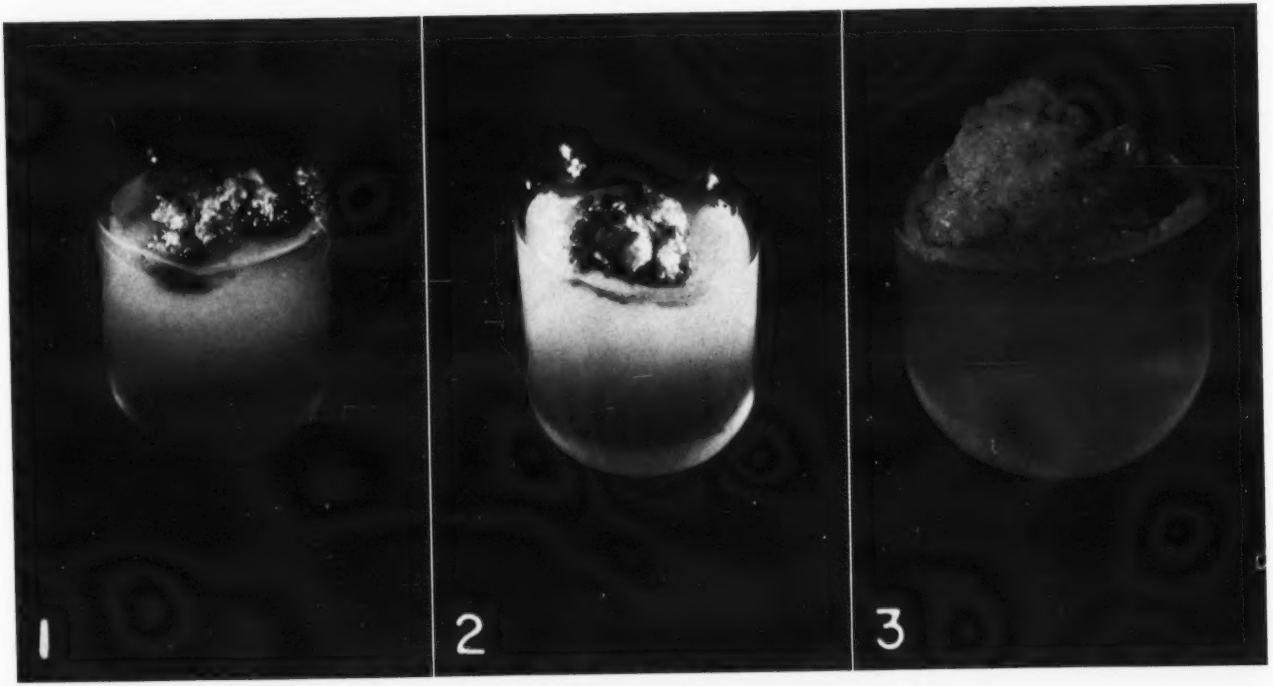
containing various amounts of the radioisotopes demonstrated that 1.0 μc/ml. was a high but nonlethal concentration. Most of the calluses survived after two months of growth upon media of that concentration of radioisotope when they were transferred to new non-radioactive media. The medium was Knop's according to Nobécourt (1937) and Gautheret (1942), but without Berthelot's microelements. Microelements were added according to Ball (1946). Thirty grams of sucrose and 1 mg. of indoleacetic acid were added per liter of medium. The radioisotope-containing substances (appropriate amounts of $H_3P^{32}O_4$ solution, or of $H_2S^{35}O_4$ solution) were added immediately before autoclaving. Calluses that had been growing upon non-radioactive medium containing autoclaved sucrose for twenty transfers (growth periods were two months each) were cut into small pieces and inoculated onto the radioisotope-containing medium. After the calluses had remained on the radioactive medium for two months they were cut into halves and transferred to non-radioactive medium and allowed to grow for two months. Reinoculations have been made subsequently in this fashion every two months. These cultures have now been grown through nine transfers on non-radioactive medium. During each of the growth periods entire calluses were removed from the culture tubes, killed, dehydrated, embedded in paraffin, sectioned and stained. Control cultures were grown upon Knop's medium with 3% autoclaved sucrose and 1 mg./l. indoleacetic acid.

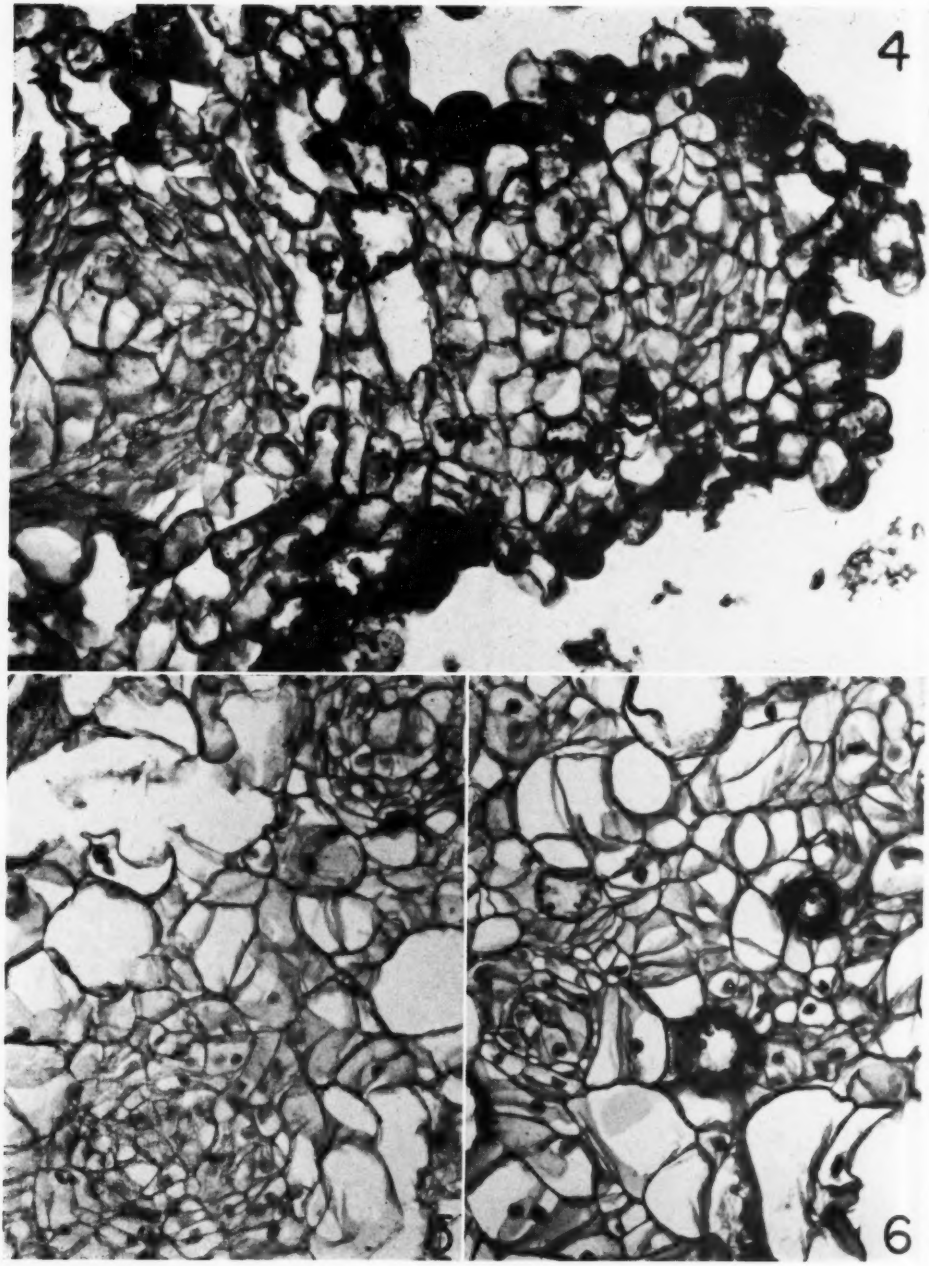
Morphology of the Radioisotope-Treated Cultures.—The growth of the calluses after the initial growth period upon radioactive medium (fig. 1, 2) has consistently been strikingly different from that of the controls (fig. 3). The former have been brown in color, due, apparently to the presence of numerous necrotic areas, and extremely rough in texture. The roughness appears to be caused by the independent growth of separate portions of an individual callus. The treated calluses were also much thicker than the controls. The immediate cause of the thickening was the great enlargement of parenchymatous cells (fig. 5, 6).

Anatomical studies show that the radioisotopes either killed or prevented the growth of many of the internal parenchymatous cells of the callus. Marginal meristems ceased to function. Growth was continued while on the radioactive medium by numerous small areas of meristematic cells (fig. 5, 6) which arose by the divisions of widely separated internal parenchymatous cells. These meristematic areas



FIGURES 1-3

Fig. 1. Callus that had been growing on normal medium seventeen months after exposure to S^{35}. The texture was very rough, and there were numerous brown areas. 1x.—Fig. 2. Callus that had been growing on normal medium seventeen months after the initial exposure to P^{32}. The rough texture and the brown areas in this callus, as in the last figure, were caused by the growth of small regions independently of each other. Intervening parenchymatous regions were crushed and became necrotic. 1x.—Fig. 3. Normal callus that had been grown during the course of the experiment entirely upon normal medium. The usual greenish-brown crust has been formed here, elevated from the agar at the center, in contact with agar at the margins. Texture of the callus is much smoother than that in the first two figures, and there are no necrotic areas since the normal growth is carried on mainly by marginal meristems. 1¼x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FIGURES 4-6

Fig. 4. Section of the margin of a normal, or control callus. At the right is the marginal meristem in which tiers of new cells have been produced. A large tracheid group surrounded by cambium occurs to the left of the marginal meristem. The dark cells are filled with tannins. 196x.—Fig. 5. Section of a callus that had grown 16 days on medium containing P^{32} at 1 μc/ml. Two meristematic areas in which new cells were being formed are seen at upper right and lower left. These meristematic areas probably were homologous with the small meristematic areas that produce the tracheid groups in the normal callus. Parenchymatous cells between the meristematic groups had greatly enlarged. 196x.—Fig. 6. Section of a callus that had grown 16 days on medium containing S^{35} at 1 μc/ml. At the center, left and right, are seen two meristematic areas in which new cells had been produced. These meristematic cells probably were homologous with the small areas that produce the tracheid groups in the normal callus. Parenchymatous cells between the meristematic groups had greatly enlarged. 196x.

appeared to be homologous with the cells that were earlier described (Ball, 1950) as giving origin to tracheid groups in the normal callus. These groups of meristematic cells in the treated calluses were, however, much more numerous than the tracheid groups of the normal controls (cf. fig. 4 with 5, 6). This method of growth continued after the initial exposure to the radioisotopes and the successive transfer of the calluses to non-radioactive medium. The fact that the half-life of S^{35} was much longer than that of P^{32} seemed not to be a factor in the subsequent growth. Thus, the calluses originally exposed to S^{35} remained radioactive for a much longer time than those exposed to P^{32}, but the patterns of growth were apparently determined by the original exposure to the internal radiation of the isotopes and the carry-over of the decreasing amounts of radioactive isotopes in the transferred calluses did not prevent the growth of the groups of meristematic cells among the internal parenchyma. In fact, it would appear that these groups of meristematic cells were the only "radioresistant" or "radioisotope-resistant" cells in the treated calluses, and the survival of the cultures depended entirely upon their continued cell divisions. In further contrast to the growth of a normal callus, however, these radioisotope-treated calluses did not immediately form tracheid groups from these internal meristematic regions. The meristematic regions (figs. 5, 6) became instead the sole growing centers of the calluses.

Growth of the calluses after the initial exposure to the radioisotope was very irregular because each of these meristematic areas was producing new cells independently of the others (fig. 7, 8). Intervening parenchymatous cells, which had apparently been so affected by the radioisotope that they could no longer divide, were crushed. They often showed in the sections as large necrotic areas between the new cells produced by the growing meristematic regions. The necrotic areas of cells between the independently growing spheres of cells were usually more prominent in cultures that had been exposed to P^{32} (fig. 9) than in those cultures that had been exposed to S^{35} (fig. 10).

The pattern of growth of calluses after treatment with radioisotopes was thus chaotic. The failure to develop marginal meristems prevented the formation of the "thin crust" pattern (fig. 3, 4) of growth characteristic of the normal callus (Ball, 1950). The correlations of growth shown in the normal callus were thus lost, and a highly irregular, non-definitive pattern obtained.

There appeared to be a decrease of cellular differentiation in calluses

after initial growth in the presence of either of the radioisotopes. There were fewer tannin-containing cells (fig. 5-10) than in the controls (fig. 4). Tracheid groups were fewer (fig. 8-10) than in the controls, and when they occurred the central part of the group consisted mostly of parenchyma; few tracheids (empty cell walls with bordered or scalariform pitting) were differentiated.

The radioisotope-treated calluses have been transferred nine times, and therefore have grown on non-radioactive medium approximately 19 months. All histological preparations made, except three, showed the chaotic growth by internal groups of meristematic cells. The exceptional three preparations showed recovery of normal growth by the activity of marginal meristems and the formation of tracheid groups in the internal parenchyma (fig. 11, 12, 13). All types of cells characteristic of the original normal callus were present in these recovered calluses. These recoveries may be considered evidence that the changes induced by the radioisotopes in this series of experiments are temporary. It may be significant that calluses treated with P^{32} showed two recoveries at the sixth transfer (14 months on non-radioactive medium, fig. 11, 13), while those treated with S^{35} have shown but a single recovery and that was in the eighth transfer (17 months on normal medium, fig. 12). Recovery of the S^{35}-treated callus appeared to be less complete than that of the P^{32}-treated ones. Fig. 12 shows that a marginal meristem (m) had only begun to produce cells, while the recovered calluses after P^{32}-treatments (fig. 11, 13) showed close approaches to the normal.

Continued growth and histological studies will be necessary to determine whether any nuclear changes have been brought about by the radioisotopes that might cause permanent modifications.

Discussion.—There appeared to be no relationship between meristematic activity of cells and their susceptibility to internal radiation. Marginal meristems and the mature internal parenchymatous cells were inactivated by the radioisotopes and ceased to divide. Meristematic cells or cambia around the tracheid groups, in which numerous divisions usually occur, were equally inactivated by the radioisotopes. However, other meristematic cells, the small groups that gave origin to new tracheid groups, continued divisions and thus formed the new tissues of the reconstituted callus. Such differences in susceptibility to the radioisotopes may be used as evidence that these meristems—marginal meristems and cambia on the one hand, and the precursors of tracheid groups on the other—are of different physiology.

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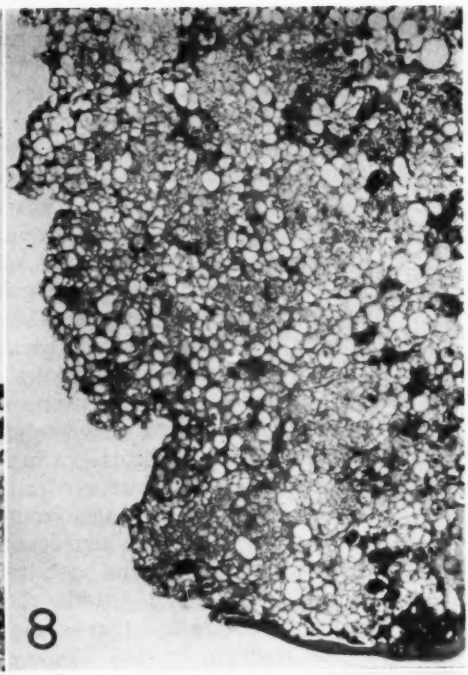
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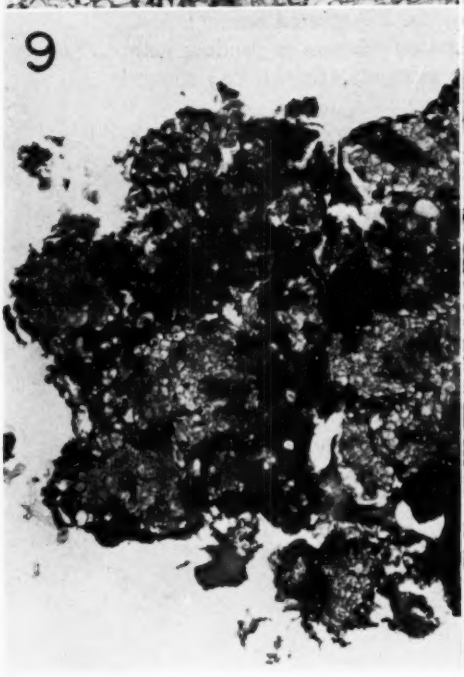
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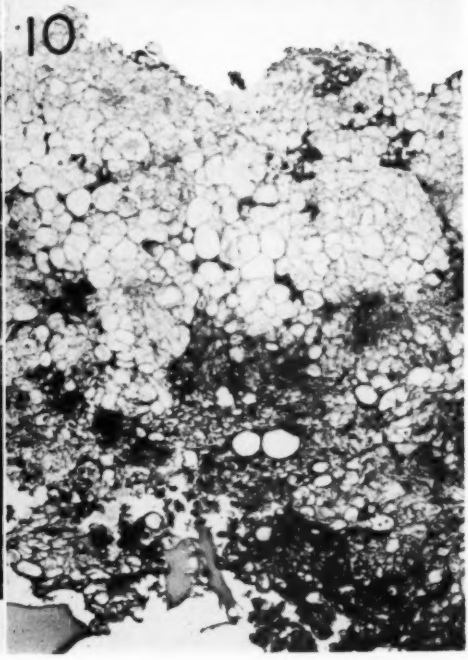
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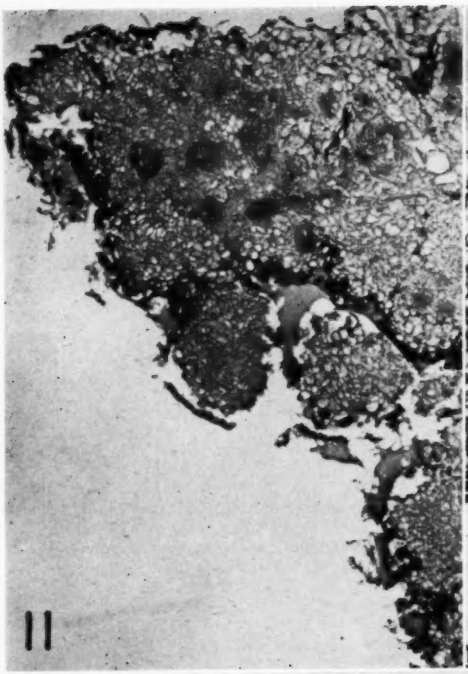
FIGURES 7-10

Fig. 7. Section of a portion of a callus that had grown six months on normal medium after the initial exposure to P^{32}. Numerous areas of small cells have been produced by the separate meristematic regions. In some cases the separate areas of new cells have attained such volume as to crush the surrounding large parenchymatous cells into necrotic areas. 28x.—Fig. 8. Section of a portion of a callus that had grown six months on normal medium after the initial exposure to S^{35}. The numerous separate meristematic areas had produced cells and increased their volumes so that some crushing of the intervening large parenchymatous cells into necrotic areas had resulted. 28x.—Fig. 9. Section of edge of a callus that had grown fourteen months on normal medium after the initial exposure to P^{32}. The numerous separate meristematic areas had produced cells and increased their volumes to such an extent that the intervening mature parenchymatous cells were crushed and became large necrotic areas. 28x.—Fig. 10. Section of a portion of a callus that had grown fourteen months on normal medium after the initial exposure to S^{35}. The masses of cells produced by the separate meristematic regions had increased their volumes. The intervening parenchymatous cells had become necrotic, but less so than in calluses after similar treatment with radioactive phosphorous. 28x.



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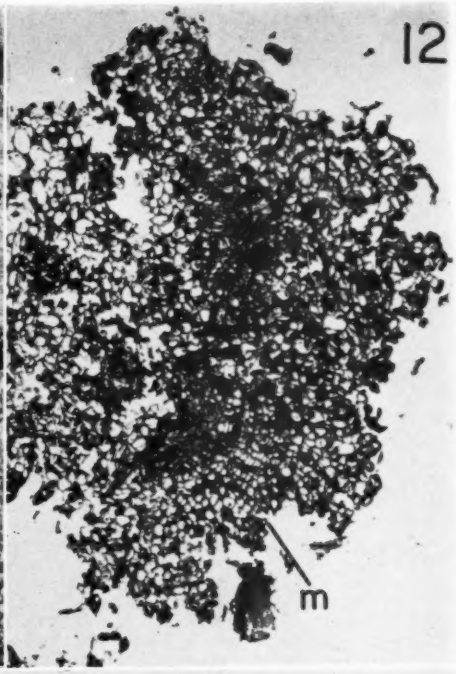

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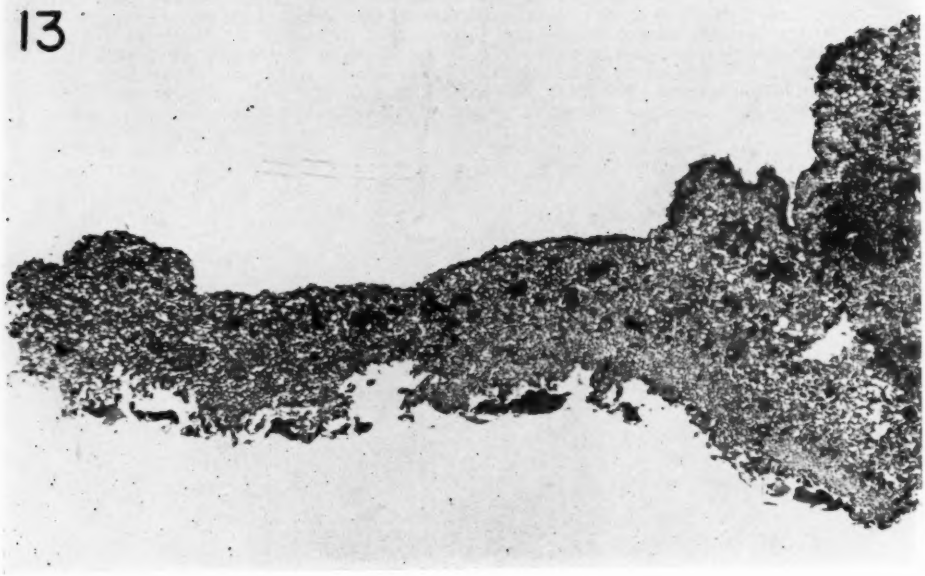

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FIGURES 11-13

Fig. 11. Section of a margin of a callus that had grown fourteen months on normal medium after the initial exposure to P^{32}. Growth in this region of this callus showed evidences of recovery to the normal. A marginal meristem had produced a continuous tract of tissue in which occurred tracheid groups surrounded by cambia. There was no growth in this recovered portion by independent groups of cells. 28x.—Fig. 12. Section of a margin of a callus grown seventeen months on normal medium after the initial exposure to S^{35}. Growth shows approaches to the normal in that a functional marginal meristem (m) occurs, and tracheid groups surrounded by cambia are found among the parenchymatous tissue. 28x.—Fig. 13. Section of a margin of a callus grown fourteen months on normal medium after the initial exposure to P^{32}. The abnormal tissue resulting from the chaotic growth of the separate meristematic areas is seen at the extreme right. The "thin crust" portion at the center and left part of the photomicrograph is the approximately normal tissue produced by a marginal meristem (extreme left). This growth represents the most nearly complete recovery of the callus after the exposure to the radioisotope. 10x.

It has been shown (Ball, 1953a) that when the callus grew on medium containing lethal amounts of these radioisotopes the marginal meristems and the cambia around the tracheid groups accumulated much of the radioisotopes. The internal mature parenchymatous cells were similarly shown to have accumulated far less of the radioisotopes. It was thought (Ball, 1953b) on the basis of the experiments of growing the callus on lethal amounts (1.5 μc/ml.) of P^{32} that the internal parenchymatous cells were less susceptible than other living cells to the effects of the radioisotope. The present results demonstrate that certain derivatives of the internal parenchymatous cells alone survived and were able to divide after treatments with sublethal doses of this radioisotope. The earlier finding (Ball, 1953b) that internal parenchymatous cells were disintegrated by lethal doses (1.5 μc/ml.) of S^{35}, while the marginal meristems appeared to be more resistant to the radioisotope, is not substantiated by the present work. The present results with S^{35} indicate that, as with P^{32}, when a sublethal amount is used, certain of the internal parenchymatous cells of the calluses alone survived and were able to divide to form new meristematic regions.

The recoveries of the calluses from the effects of the radioisotopes are of significance. They demonstrated that the "radioisotope-resistant" cells contained in themselves the potentiality of producing the various kinds of cells characteristic of the normal callus. A comparable result was reported by Vöchting (1908) who showed that from the pith of the tuber of kohlrabi all the primary tissues of the tuber can be produced.

All the types of cells were not differentiated in the isolated spheres of cells that grew in the treated calluses. Only after recoveries of the normal growth pattern (fig. 11, 13) were all the types of cells found. It is a logical interpretation that such cell types as the tannin-filled cells, the tracheids, meristematic cells at margins and around the tracheid groups, could be differentiated only because the callus had recovered its normal pattern, i.e., had begun again to grow as a unit.

Since it was shown (Ball, 1953a) that the marginal meristems and cambia of the tracheid groups accumulate far more of both of the radioisotopes than the internal parenchyma, it is difficult to say whether the meristems are more, or less resistant to radiation than the parenchyma. The fact that with a lethal amount of both of these radioisotopes all cells of the calluses were killed, and in contrast, with

a sublethal amount of them only some derivatives of certain internal parenchymatous cells survived, however, gives a practical measure of radiation resistance. On this basis the present results disagree with the suggestion of Bergonié and Tribondeau (1906) that animal cells capable of division are more susceptible to radiation than are mature, differentiated cells. The initial and continued resistance of the internal meristematic groups of cells to the radioisotopes is evidence that certain meristems, in spite of their recurrent mitoses and cell divisions, are radioresistant. It is important to emphasize that only certain meristems may have this radioresistant quality, for the original meristems of the calluses proved susceptible. Bloom's (1947) thesis that mitosis may not be a predisposing factor toward radiation damage would appear to be substantiated at least in this case.

After the treatment with radioisotopes the continued growth of the callus by the divisions of certain groups of cells was a phenomenon of replacement of killed tissues. It is thus a special case of wound healing and regeneration. It is comparable to the situation described by Tullis (1949) for animals exposed to atomic bomb radiation where replacement of radiosensitive cells can be accomplished by certain radioresistant uninjured stem cells.

Summary

Calluses of *Sequoia sempervirens* treated with a sublethal amount of P^{32} or S^{35} showed failure of all cells to proliferate except the new initials of tracheid groups. These meristematic groups built up, after several growth periods on non-radioactive medium, large masses of tissue separated from each other by necrotic material. Eventually some of the treated calluses showed recoveries in the direction of growth by marginal meristems and the formation of the normal component of tissues. The isolated groups of radioisotope-resistant meristematic cells thus were capable of producing all the cell types of the normal callus. The fact that these meristematic cells, in spite of their earlier demonstrated high absorption of the radioisotopes, were resistant to the radiation while mature parenchyma and other cell types were not, has been utilized as evidence that cells undergoing mitosis and cytokinesis are not necessarily highly radiosensitive.

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THE RELATIONSHIP OF UPTAKE OF P^{32} TO MITOSIS

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Injections of tissue suspensions stimulate mitotic activity in homologous organs of the rat (*Teir* 1952a, 1952b, *Teir* and *Ravanti* 1953). The stimulation, however, was only observed in some of the cases, while in others the mitotic rate did not exceed that of the controls. In order further to investigate this point, in our present experiments we used P^{32} as an indicator of the phosphorus consumption in organs where mitotic stimulation would be expected. We also wished to investigate whether the mitosis-stimulating activity is tissue specific, as was indicated by some of our previous experiments.

Methods

Rats of the Wistar strain about 6 weeks old were used. The tissue suspensions were derived from the liver and the orbital gland of 2-week-old rats. The concentration of the orbital gland suspension was 0.5 mg./ml., that of the liver suspension 3 mg./ml. and the quantity injected was 1 ml. The controls received the same amount of distilled water. The time of influence of the suspensions varied between one and three days. The concentration of the P^{32} solution was 5-7 μC/ml. and amount injected 1 ml. The P^{32}solution was injected exactly one hour before decapitation. All injections were given intraperitoneally. Samples were taken from the liver and the orbital gland for the determination of radioactivity with the method used by *Lamberg* (1953). The radioactivity was determined in counts per minute/mg. fresh weight. The total weight of the orbital gland and the liver was measured for the calculation of the total radioactivity. The mitoses were counted per hundred fields of vision in sections fixed in Bouin and stained in Heidenhain's hematoxylin.

Results

Experiment 1. For this experiment 18 rats were used. They were divided into three groups of 6 rats. The first group received distilled water, the second orbital gland suspension and the third liver suspension two days before decapitation. The amount of P^{32} was 5 μC to all rats.

The uptake of P^{32} by the orbital gland and the liver was greater in all tissue suspension groups than in the control group (Table 1).

TABLE 1

Uptake of P^{32} by the Orbital Gland and the Liver

Experiment 1. The radioactivity is given as radioactivity of the sample in counts/min./mg. fresh weight (upper rows in bold face type) and as total radioactivity of the organ in counts/min. (lower rows in Roman type). The percentage figures are calculated taking the controls (distilled water) as 100%.

		Organ studied			
		Orbital gland		Liver	
		Radioactivity of sample c/min./mg.		Radioactivity of sample c/min./mg.	
Material injected	Number of rats	Total radioactivity c/min. Mean ± S.D.	%	Total radioactivity c/min. x 10^{-3} Mean ± S.D.	%
Distilled water	6	**17.4± 1.0**	**100**	**92.2± 5.0**	**100**
		1460±137	100	369±14.5	100
Orbital gl. suspension	6	**19.8± 2.0**	**114**	**93.7± 7.2**	**100**
		1596±152	109	372± 9.4	101
Liver suspension	6	**18.8± 2.0**	**108**	**113.2± 9.0**	**123**
		1427±136	98	437±15.3	118

The increase was most clear in the orbital gland in the group treated with orbital gland suspension and in the liver of the group treated with liver suspension. Only the last-named difference of the total uptake of P^{32} by the liver could be proved statistically significant.

The count of mitoses did not indicate a mitotic stimulation in this experiment (Table 2). The mitotic values are so low that no certain conclusions can be drawn of a possible correlation between mitosis and uptake of P^{32} in the organs studied.

Experiment 2. For this experiment a total of 66 rats were used. The rats were divided and treated in the same way as in the first experiment but the rats were killed after one, two and three days in each group. The amount of P^{32} was 7 μC. Half of the control rats did not receive any radioactive phosphorus at all.

TABLE 2

THE MITOTIC RATES. EXPERIMENT 1

Material injected	Number of rats	Organ studied: Orbital gland Mean + S.D.	Organ studied: Liver Mean + S.D.
Distilled water	6	3.8±0.9	1.5±0.6
Orbital gl. suspension	6	1.8±0.7	1.2±0.5
Liver suspension	6	1.3±0.2	0.7±0.3

Determination of radioactivity (Table 3) showed that the result in the one- and two-day groups was much the same as in the first experiment. In the three-day groups only the liver suspension-treated group showed an increased uptake of P^{32} by the liver when calculated on the basis of the total radioactivity of this organ. It must, however, be considered that the specific increase was in no case statistically significant.

TABLE 3

EXPERIMENT 2. EXPLANATION OF THE TABLE IS THE SAME AS IN TABLE 1

Duration of exper. days	Material injected	Number of rats	Organ studied: Orbital gland Radioactivity of sample c/min./mg. Total radioactivity c/min. Mean+S.D.	%	Organ studied: Liver Radioactivity of sample c/min./mg. Total radioactivity c/min. x 10^{-3} Mean+S.D.	%
1	Distilled water	3	**22.1± 3.3** 1700±313	**100** 100	**126± 1.3** 467± 7.7	**100** 100
	Orbital gl. suspension	6	**25.0± 1.1** 2570±229	**113** 151	**122± 4.2** 511± 7.5	**97** 110
	Liver suspension	6	**22.1± 0.9** 2340± 67	**100** 138	**115± 1.3** 508±16.1	**91** 109
2	Distilled water	3	**26.8± 2.4** 1890±159	**100** 100	**136± 9.7** 479± 9.0	**100** 100
	Orbital gl. suspension	6	**22.8± 0.9** 2020±101	**85** 107	**125± 7.9** 485±21.8	**92** 101
	Liver suspension	6	**18.6± 0.5** 1950± 89	**69** 103	**118± 7.9** 531±24.1	**87** 111
3	Distilled water	3	**27.7± 0.6** 2010±127	**100** 100	**137± 5.4** 448± 5.8	**100** 100
	Orbital gl. suspension	6	**21.4± 1.2** 1930±191	**77** 96	**112± 2.5** 472± 9.7	**82** 105
	Liver suspension	6	**21.8± 1.0** 2040±132	**79** 101	**124± 6.7** 530±13.2	**91** 118

The mitotic rate is indicated in Table 4, in which the P^{32} series and the suspension series without P^{32} have been combined since no significant difference could be established between them. The mitotic values are throughout higher in this experiment than in the previous one. In the orbital gland suspension series the mitotic rate in the orbital gland is considerably higher than in the controls. The per cent of significance of the result is 92. In the liver, the mitotic rate in either suspension series did not differ from those of the controls.

TABLE 4
THE MITOTIC RATES. EXPERIMENT 2

		Duration of experiment days					
		1		2		3	
Organ studied	Material injected	Number of rats	Mean ± S.D.	Number of rats	Mean ± S.D.	Number of rats	Mean ± S.D.
Orbital gland	Distilled water	3	7.0±1.0	6	7.7±2.6	3	3.7±0.8
	Orbital gl. suspension	9	20.3±4.5	8	10.5±2.5	9	5.9±1.3
	Liver suspension	9	10.1±1.9	9	8.9±1.9	9	1.8±0.3
Liver	Distilled water	3	5.3±0.6	6	4.2±1.3	3	1.7±0.8
	Orbital gl. suspension	9	2.1±0.6	9	6.4±1.0	9	2.1±0.4
	Liver suspension	9	5.0±1.0	9	4.7±1.0	9	2.2±0.4

A correlation was found between the mitotic rate and the corresponding uptake of P^{32} by the orbital gland (fig. 1.). The correlation is clearer when all three orbital gland series are combined (fig. 2).

With regard to the liver mitoses, no clear correlation between mitotic rate and the uptake of P^{32} could be established. This result seems quite logical, considering that the uptake of P^{32} by the liver is no doubt more influenced by other factors than the sparse mitoses.

CONCLUSIONS

In the orbital gland in the second experiment, in which mitotic stimulation was definitely observed by tissue suspension injections, there was a clear correlation between the mitotic rate and the uptake of P^{32}. This fits well with the general view that growing tissues whether normal or malignant, show a characteristically higher uptake of P^{32} (reviewed by Hevesy 1948 and *Heidelberger* 1953). In their

studies on the P^{32} uptake in tissue cultures *Hull* and *Kirk* (1950) arrived at the conclusion that "strong indications are found that the incorporation of inorganic phosphorus into desoxyribonucleic acid parallels the mitotic coefficient and serves as a sensitive index of the synthesis of new cells". This assumption seems to have been confirmed as regards the orbital gland. In our controls, no correlation whatsoever could be observed. It is possible that there is a clear

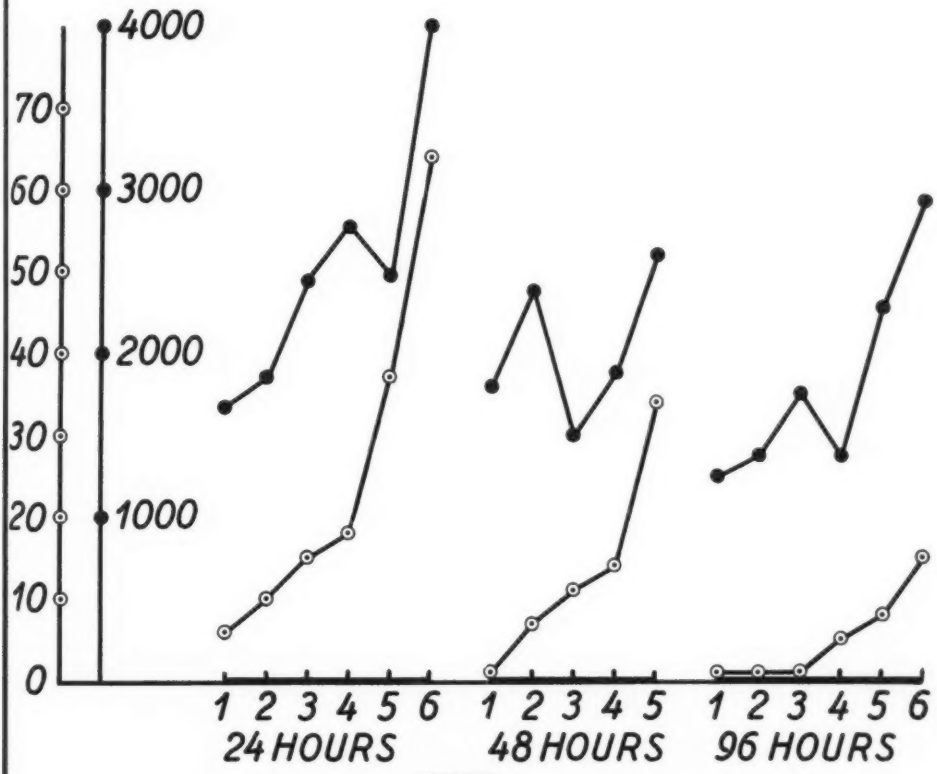

FIGURE 1

Experiment 2. Correlation between mitotic rate and uptake of P^{32} by the orbital gland 24, 48, and 96 hours after injection of orbital gland suspension. The inferior curves (corresponding scale on the left) represent the individual mitotic rates, the superior curves (corresponding scale on the right) represent the individual uptake values of P^{32}.

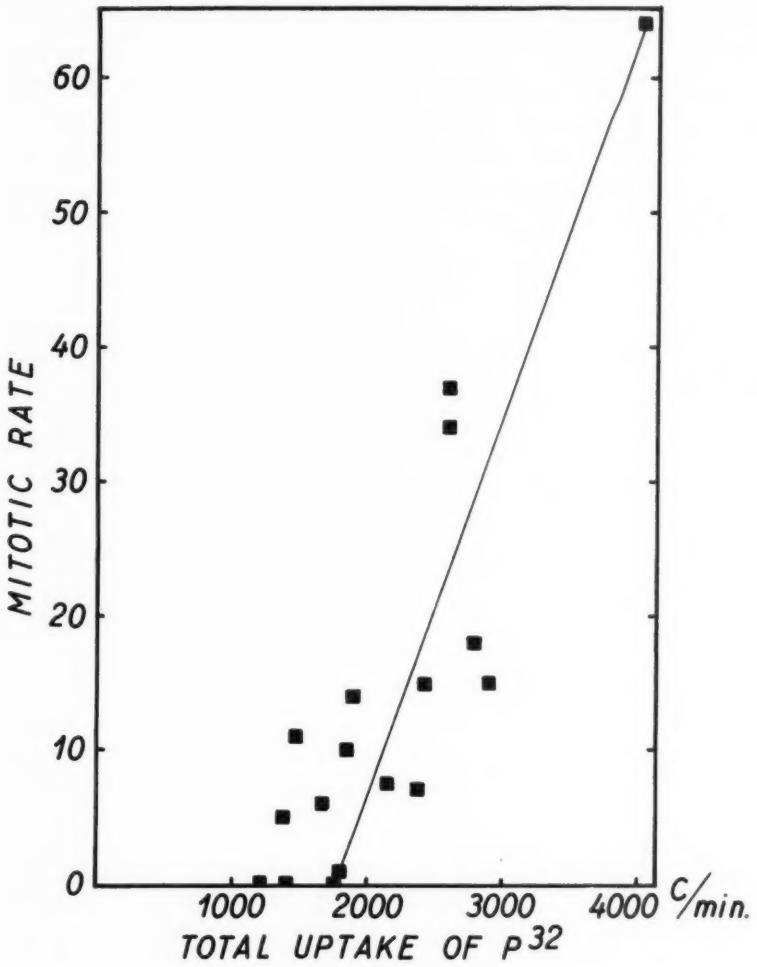

FIGURE 2

Experiment 2. Correlation between mitotic rate and uptake of P^{32} by the orbital gland after injection of orbital gland suspension. All three series are combined.

correlation between the P^{32} uptake and mitotic rate only if the mitotic rate is high either on account of tissue suspension stimulation or for some other reason.

In the liver no correlation could be established in our investigations. It should be mentioned, however, that in the present experiments the liver suspensions did not induce any mitotic stimulation in the liver. Since, however, a definite increase in the uptake of P^{32} by the liver can be noticed in the series treated with the liver suspension, this is obviously due to factors other than an increased mitotic rate. *Marshak* (1940, 1941) has shown that regenerating liver incorporates P^{32} in the nuclei (DNA fraction) at the time when mitoses are most frequent.

We have tried to solve the problem of the tissue specificity of the mitosis-stimulating factor, using tissue suspensions labelled with P^{32} (*Kuusi, Isotalo* and *Teir* 1953). Such a specificity could not be conclusively proved. Our present experiments argue in favour of tissue specificity. A mitotic stimulation could be established in the orbital gland most clearly one day after injection of orbital gland suspension. At the same time there was a slight stimulation caused by liver suspension. The corresponding values for the uptake of P^{32} show that here, too, the orbital gland caused a greater increase and the liver suspension a smaller one. The specificity is thus not absolute but relative.

Summary

1. Mitotic rate and uptake of P^{32} was studied in the outer orbital gland and liver of the rat after injection of tissue suspension of these organs.

2. When mitotic stimulation was observed, a correlation was found between mitotic rate and uptake of P^{32} in the orbital gland. In the liver an increased uptake of P^{32} was found without mitotic stimulation after injection of tissue suspensions.

3. In the action of tissue suspensions some organ specificity was found in respect to mitosis and the uptake of P^{32}.

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TABLE OF CONTENTS

Paper No.		Page
442	The nutritional requirements of *Acetobacter*. III. Amino acid requirements of *Acetobacter xylinum* . . By BYRON S. TEPPER AND WARREN LITSKY	193
443	Inhibition of the utilization of Glutamine by *Lactobacillus arabinosus* . . By PADMASINI AYENGAR AND EUGENE ROBERTS	201
444	Modifications in a callus culture of *Sequoia sempervirens* after growth on P^{32} and S^{35} . . By ERNEST BALL	215
445	The relationship of uptake of P^{32} to mitosis . . By A. ISOTALO, T. KUUSI, AND H. TEIR	229